Express Mail Label # TB546206034US

UVI-005CP2

RECOMBINANT TRANSFERRINS, TRANSFERRIN HALF-MOLECULES AND MUTANTS THEREOF

INBAT

Related Applications

This application is a continuation-in-part of U.S. Application Serial Number 07/832,029 filed February 6, 1992, which is now ~~pending~~ abandoned and which is a continuation-in-part of U.S. Application Serial Number 07/652,869 filed February 8, 1991, now abandoned.

Government Support

The work leading to this invention was supported by one or more grants from the United States Government.

Background of the Invention

The iron-binding pseudoglobulins collectively called transferrins or siderophilins comprise a class of proteins with strikingly similar features. X-ray crystallographic analyses of human lactoferrin (Anderson, B.F. *et al.* (1987) Proc. Natl. Acad. Sci. USA 84:1769-1773) and rabbit serum transferrin (Bailey, S. *et al.* (1988) Biochemistry 27:5804-5812) reveal that these proteins consist of two similar lobes connected by a short bridging peptide and that each lobe contains two domains defining a deep cleft containing the binding site for a metal ion and a synergistic anion.

The chicken ovotransferrin gene has been expressed in transgenic mice (McKnight, G.S. *et al.* (1983) Cell (Cambridge, MA) 34:335-341) and a fusion protein of part of rat transferrin with galactosidase has been expressed in *E. coli* (Aldred, A. et al. (1984) Biochem. Biophys. Res. Commun. 122:960-965). Except for this fusion protein, attempts to express transferrin or portions of the molecule in prokaryotic systems have been unsuccessful (Aldred, A. *et al.* (1984) Biochem. Biophys. Res. Commun. 122:960-965). The highly convoluted structure of the protein and large number of disulfide bridges in the molecule are probably the major impediments to expression in bacterial hosts. Attempts to mimic partially the natural protein folding environment by targeting the protein for bacterial membrane transport via an attached alkaline phosphatase signal sequence have been unsuccessful.

Summary f the Invention

This invention pertains to recombinant transferrin, to recombinant transferrins that bind to the transferrin receptor, to recombinant transferrin half- molecules comprising at least the metal-binding domains of a single lobe (amino-terminal or carboxy-terminal) of transferrin and to stable cell culture system for expression of the transferrin. The recombinant transferrin can be expressed in stable, transformed eukaryotic cells, such as baby hamster kidney cells, to yield essentially homogeneous (monodisperse) preparations of the full or half-molecule forms. The invention also pertains to mutant transferrins, non-glycosylated transferrins and transferrin half-molecules which have metal-binding or other properties which are different from the natural (wild-type) form of the transferrin. These include mutant transferrins and transferrin half-molecules which bind iron or other metals more or less avidly than natural transferrin.

Transferrin half-molecules can be used in metal chelation therapy to treat individuals affected with abnormalities of metal regulation or with metal poisoning. For example, transferrin half-molecules, especially mutant forms which bind iron with a higher avidity than natural transferrin, can be administered to iron-overloaded individuals, e.g., thalassemics, in order to clear excess toxic iron from their bodies. In addition, half-molecules, or mutants thereof having altered metal ion selectivities, could be used to clear other toxic metals, e.g., lead, mercury, cadmium, copper and zinc from the body.

Description of the Figures

Figure 1 shows construction of the hTF/2N expression vector in pNUT. A 2.3-kb cDNA encoding human serum transferrin was isolated from a human liver cDNA library and a 1.5-kb PstI/HaI fragment containing the complete amino-terminal domain coding sequence was cloned into M13mp18. Double translational stop codons and a HindIII recognition sequence were introduced by site-directed mutagenesis, allowing the isolation of a BamHI/HindIII fragment which, when joined to a BamHI/HpaII fragment, encodes the amino-terminal domain and signal sequence. This fragment was cloned into the eukaryotic expression vector pNUT, giving the vector pNUT-hTF/N2. In this plasmid, the transferrin cDNA is under the control of the metallothionein promoter (MT-1 pro) and the human growth hormone transcription termination signals (hGH3'); pNUT also contains the SV40 early promoter (SV40) driving expression of a resistant DHFR cDNA (DHFR cDNA) using transcription termination signals from human hepatitis B virus (HBV).

Figure 2 shows a Western blot of immuno- precipitates from various baby hamster kidney cell lines. Samples of cell lysates (a) and medium (b) from Zn-induced cell cultures were precipitated with anti-hTF antiserum. Samples of the resuspended pellets were analyzed by NaDodSO$_4$-PAGE, transferred to nitrocellulose and developed with anti-hTF antiserum followed by alkaline phosphatase conjugated anti-IgG. The hGH-pNUT and hTF/2N-pNUT transformed cell lines were selected in 500 μM MTX and all

cell culture was performed in DMEM/10% fetal calf serum. Lane 1, BHK cells; lane 2, hGH-pNUT transfected BHK cells; lane 3, hTF/N2-pNUT transfected BHK cells. The positions of molecular weight markers (x 10^{-3}) are indicated to the right of the blot, the position of the additional protein band of M_r 37,000 is also indicated (<37) to the right of the blot.

Figure 3 shows the isolation and PAGE analysis of hTF/2N. (Panel A) FPLC isolations on a column of Polyanion SI of recombinant hTF/2N (upper trace) and proteolytically derived hTF/2N (lower trace). (Panel B) NaDodSO$_4$-PAGE (5-12% gradient of acrylamide) of molecular weight standards (lane Mr) and 3 μg of each of peaks a-d from panel A. (Panel C) Urea-PAGE under nonreducing conditions of the FPLC peaks a-d (recombinant hTF/2N species) and peaks e-h (proteolytically derived hTF/2N species) from panel A. The positions of the apo-protein (apo) and iron-bound protein (Fe) are indicated. The conditions used for FPLC are given under Materials and Methods. FPLC fractions were pooled as follows; peak a (fractions 23-27), peak b (28-31), peak c (32-38), peak d (39-45), peak e (28-31), peak f (32-36), peak g (38-44), and peak h (46-51).

Figure 4 shows titration of the major form recombinant hTF/2N with 10 mM Fe(III)(NTA)$_2$. The amount of protein was 3.68 A_{280} units in 1.00 mL of 10 mM $NaHCO_3$. Visible spectra were run 5-10 minutes after each addition of iron to the magnetically stirred cuvette.

Figure 5 shows proton magnetic resonance spectra of recombinant hTF/2N. (a) Fourier transform spectrum with a line broadening of 2 Hz. (b) Convolution difference spectrum with a line broadening of 4 Hz and DC = 4.0, NS = 68,500. The protein sample was 8 mg in 0.1 mL of 0.1 M KCl in ^2H_2O.

Figure 6 shows the ^{19}F nuclear magnetic resonance spectrum of m-F-Tyr recombinant hTF/2N. The figure shows a Fourier transformation with a line broadening of 10 Hz, NS = 30,000. The protein sample was 6 mg in 0.1 mL of 0.1 M KCl in ^2H_2O; the reference was 0.1 M trifluoroacetic acid in ^2H_2O.

Figure 7 shows two separate oligonucleotides used as PCR primers to create the hTF/2C coding sequence. An *EcoRI* restriction fragment including coding sequence for the entire carboxy lobe was used as a template for 25 rounds of PCR amplification. Oligonucleotide 1 includes a *Sma*I recognition site and the natural hTF signal sequence at its 5' end and matches the coding sequence for amino acids 334 -341 of hTF at its 3' end. Oligonucleotide 2 matches sequence in the 3' untranslated region of the hTF cDNA and introduces a second *Sma*I recognition sequence at this site.

Figure 8 shows the construction of the hTf N413D/N611D expression vector in pNUT. Using a plasmid called pUC2-3 which contains the DNA coding region for the C-terminal lobe of hTf, each of the two mutagenic oligonucleotides described in Example V was used separately to introduce the desired mutations. The two resulting plasmids, Tf-

N413D and Tf-N611D, were cut with *Acc*I and *Stu*I; the DNA fragments containing the mutated residues were removed from agarose gel slices and ligated into the *Acc*I site of a full-length Tf cDNA clone in pUC19 to give hTf(N/D). This plasmid was cleaved with *Sac*I and *Sph*I, the ends were made blunt, and the fragment was cloned into the *Sma*I site of pNUT to give pNUT-hTf(N/D). In this plasmid, the cDNA is under the control of the metallothionein promoter (MT) and the human growth hormone transcription termination signals (hGH). pNUT also contains the SV40 early promoter (SV40) driving expression of a mutated form of the dihydrofolate reductase (DHFR) cDNA using transcription termination signals from human hepatitis B virus (HBV).

Detailed Description of the Invention

This invention provides for the production of recombinant transferrin, recombinant transferrin half-molecules and mutant forms of full-length transferrin and transferrin half-molecules which have altered properties, such as improved metal-binding capability, compared to the natural transferrin molecules. Recombinant transferrins can be produced in large quantities and in substantially homogeneous (monodisperse) form. For example, recombinant half-molecules of human serum transferrin can be produced as an essentially homogeneous preparation substantially free of other human serum proteins. In contrast, half-molecules prepared by proteolysis of the holo-protein are difficult to purify and, in fact, the carboxy-terminal half of human transferrin cannot be satisfactorily prepared by proteolytic means. Recombinant techniques also allow the application of mutagenesis to design and produce new forms of transferrin.

In general, a recombinant transferrin of this invention is produced by transfecting a suitable host cell with a nucleic acid construct encoding the transferrin, culturing the transfected host cell under conditions appropriate for expression and recovering the recombinant transferrin expressed by the cell. The amino acid sequences for eight transferrins have been reported (See S.S. Baldwin Comp. Biochem Physiol. 106b: 203-218 (1993)). The DNA sequence (SEQ ID NO: 1) and amino acid sequence (SEQ ID NO: 2) for human serum transferrin has been determined (Yang, F. *et al.* (1984) Proc. Natl. Acad. Sci. USA 81:2752-2756). Full-length DNA for production of recombinant transferrins or truncated DNA encoding either the amino-terminal or carboxy-terminal lobe of transferrin or a portion thereof can be obtained from available sources or can be synthesized according to the known sequences by standard procedures. In order to provide for secretion of the recombinant transferrin into cell culture medium, DNA encoding a transferrin signal sequence (or other signal sequence suitable for the expression system) is positioned upstream of the transferrin encoding DNA.

Through receptor-mediated endocytosis, cell-surface transferrin receptors deliver transferrin with its bound iron to peripheral endosomes where the iron is released into the cell and then the iron-free transferrin or apotransferrin is recycled to the extracellular fluid.

Accordingly, another aspect of the invention is a homogenous preparation of human transferrin that is recognized by a transferrin receptor and is free of other human proteins.

Mutant forms of transferrin and transferrin half-molecules can be produced by standard techniques of site-directed mutagenesis. See Taylor *et al.* (1985) Nucleic Acids Res. 13;8749-8764; Zoller, M.J. and Smith, M. (1983) Meth. Enzymol 100:458-500. In particular, mutagenesis can be used to produce mutant transferrins which have metal-binding properties that are different from natural transferrin. For example, mutants capable of binding iron more avidly than natural transferrin can be produced. To produce such mutants, metal-binding domains can be mutagenized to replace one or more amino acids involved in binding with different amino acids. In human serum transferrin, the amino acids which are ligands for metal chelation are shown below (the number beside the amino acid indicates the position of the amino acid residue in the primary sequence where the first valine of the mature protein is designated position 1)

Amino terminal lobe (amino acids 1-337)		Carboxy terminal lobe (amino acids 343-679)	
Aspartic acid	63	Aspartic acid	392
Tyrosine	95	Tyrosine	426
Tyrosine	188	Tyrosine	517
Histidine	249	Histidine	584

In other types of transferrin, the numbering is different, but the ligands (amino acids) are the same.

Other regions of transferrin control binding and these too can be targeted for mutagenesis. These are usually positively charged amino acids such as lysine, histidine or arginine. For example, a mutant transferrin half-molecule which binds iron more avidly than natural transferrin can be produced by replacing the lysine residue at position 206 with glutamine (AAG→CAG) or by replacing the histidine residue at position 207 with glutamic acid (CAG→GAG).

Further, human serum transferrin contains two N-linked oligosaccharides at Asn-413 and Asn-611 corresponding to AAT and AAC, respectively. These glycosylation sites can be removed by changing the codons to GAT and GAC which correspond to aspartic acid using, for example, oligonucleotide-directed mutagenesis. Thus, a non-glycosylated transferrin can be produced recombinantly.

The transferrin-encoding DNA is cloned into a eukaryotic expression vector containing appropriate regulatory elements to direct expression of the DNA. A preferred eukaryotic expression vector is the plasmid pNUT described by Palmiter, R.D. *et al.* (1987) Cell 50:435-443. This plasmid contains the mouse metallothionein promoter which induces transcription of the transferrin encoding DNA in the presence of heavy

metal and transcription termination signals of human growth hormone. In addition, pNUT contains dihydrofolate reductase gene under control of the SV40 early promoter with transcription termination signals from human hepatitis B virus to allow selection in cell culture. The gene encodes a mutant form of the enzyme which has a 270-fold lower affinity for the competitive inhibitor methotrexate. This allows for the immediate selection of transfected cells in very high concentrations (0.5 mM) of methotrexate and abrogates the need for a recipient cell line that is deficient in dihydrofolate reductase. pNUT also contains pUC18 derived sequences which allows it to be amplified in *E. coli* to provide sufficient amounts of the plasmid for transfection of recipient cells.

The expression vector containing the DNA encoding the transferrin is incorporated into an appropriate host cell. The preferred host cell is a eukaryotic cell which can be transformed with the vector to yield a stable cell line which expresses a functionally active transferrin construct. A particularly useful cell is the baby hamster kidney cell. Baby hamster kidney cells can be transfected with a vector carrying the DNA construct encoding a transferrin (such as the pNUT plasmid) to provide a stable cell culture system which expresses and secretes a functionally active transferrin (full or half-molecule). These cells are well-suited for economical, large scale growth and can be obtained from readily available sources.

Standard techniques, such as calcium phosphate coprecipitation or electroporation can be used to transfect the eukaryotic host cell with the vector. The cell is then cultured under conditions appropriate to induce expression of the transferrin. For example, baby hamster kidney cells transfected with the pNUT vector are stimulated to express the transferrin construct in the presence of heavy metals. Baby hamster kidney cells are preferably cultured in the medium Dulbecco's Modified Eagle's medium-Ham's F-12 nutrient mixture with the serum substitute Ultroser G™ (Gibco) at about 1%.

After an appropriate culture period, the expressed and secreted transferrin can be recovered from the culture medium. Standard purification procedures can be employed to yield a substantially homogeneous preparation of the recombinant transferrin. In one embodiment, the transferrin in the culture medium is saturated with iron and then purified by anion exchange chromatography.

The recombinant transferrins of the invention can be used to chelate and clear iron or other toxic metals from the body. The customary approach to iron chelation *in vivo* has been to assess a wide variety of naturally-occurring siderophores of microbial origin and synthetic iron chelators for their physiological effects, primarily the ability to bind and clear iron from the body. Many such compounds have been studied with varying abilities to clear iron and often with unacceptable side effects (Pitt, C.G. *et al.* (1979) J. Pharm. Exp. Therap. 208:12-18). As a result, the only iron chelator used for clearing excess iron from humans remains deferoxamine, a cyclic peptide from *Streptomyces pilosis*.

A preferred transferrin for iron chelation therapy is a mutant transferrin half-molecule which binds iron more avidly than natural transferrin. The use of a mutant half-molecule allows for more efficient chelation and removal of the metal. A particularly preferred mutant half-molecule is K206Q, described in the Exemplification below, which contains a glutamine rather than a lysine at position 206.

A transferrin half-molecule is advantageous because unlike the holo-proteins, it passes through the glomeruli of the kidney and is excreted in the urine, so that metal is not only chelated but also cleared from the body. Moreover, the single half-molecules do not bind to transferrin receptors on the membrane of tissue cells and therefore do not deliver iron to these tissues. Further, half-molecules of human transferrin would probably be recognized as "self" by the human body and therefore would not elicit an immunological response.

In addition, mutant half-molecules can be designed to have altered metal ion selectivities. The chelators could be used to clear other toxic metals from the body, e.g., lead, mercury, cadmium, and copper.

For chelation therapy, the recombinant transferrin is administered to a patient in amounts sufficient to chelate the metal and reduce circulating levels below toxic levels. Generally, it is administered in a physiologically acceptable vehicle, such as saline, by a parenteral route (typically intravenously).

Recombinant full-length human transferrin can be used in nonserum supplements or replacements for cell culture media. Transferrin is required for iron uptake by growing cells. The use of recombinant transferrin avoids the risk of contamination with, e.g., HIV or hepatitis virus associated with transferrin purified from human serum or prions from fetal bovine serum.

The invention is illustrated further by the following exemplification:

EXEMPLIFICATION

I. Production of Recombinant Transferrin Half- Molecule Comprising the Amino-Terminal Lobe.

MATERIALS

T4 DNA ligase, DNA polymerase I (Klenow fragment) and T4 polynucleotide kinase were purchased from Pharmacia-PL Biochemicals. Restriction endonucleases were purchased from Pharmacia-PL Biochemicals and Bethesda Research Laboratories. Oligodeoxyribo- nucleotides were synthesized on an Applied Biosystems 380A DNA Synthesizer. Nitrocellulose filters were obtained from Schleicher and Schuell, ^{32}P-labeled nucleotides from New England Nuclear, goat anti-human transferrin antiserum from the Sigma Chemical Company, formalin-fixed *Staphylococcus aureus* cells from Bethesda Research Laboratories, the Protoblot immunoscreening detection system from Promega, the oligonucleotide-directed mutagenesis kit from Amersham, Dulbecco's modified essential medium and fetal bovine serum from Gibco, and anti-human transferrin monoclonal antibody HTF-14 was from the Czechoslovakian Academy of Sciences. All other reagents were analytical grade or purer.

METHODS

Isolation of Human Serum Transferrin (hTF) cDNA. A human liver cDNA library constructed in the *E. coli* expression vector pKT-218 (Prochownik, E.V. *et al.* (1983) J. Biol. Chem. 258:8389-8394) provided by Dr. Stuart Orkin, (Harvard University) was screened using a synthetic oligonucleotide coding for the amino-terminal eight amino acids of serum hTF as a hybridization probe. The oligonucleotide corresponded to nucleotides 88 to 111 of the hTF cDNA sequence reported by Yang, F. *et al.* (1984) Proc. Natl. Acad. Sci. USA 81:2752-2756). The oligonucleotide was end-labeled with T4 polynucleotide kinase and ^{32}P-ATP (Chaconas, G. and van de Sande, J.H. (1980) Methods Enzymol. 65:75-85), and used to screen approximately 10^5 colonies. Restriction endonuclease mapping of positive clones and DNA sequence analysis were performed by using standard procedures with pUC19 and M13mpl9 vectors, respectively (Maniatis, T. *et al.* (1982) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY; Messing, J. (1983) Methods Enzymol. 101:20-78; Sanger, F. *et al.* (1977) Proc. Natl. Acad. Sci. USA 74:5463-5467).

Expression Vector and Cell Culture. The eukaryotic expression vector pNUT (Palmiter, R.D. *et al.* (1987) Cell (Cambridge, MA) 50:435-443) and baby hamster kidney (BHK) cells were provided by Dr. Richard D. Palmiter (Howard Hughes Medical Institute, University of Washington). After synthesis, oligonucleotides were purified on C_{18} reverse-phase columns (Sep-Pak, Waters Associates; Atkinson, T. and Smith, M. (1984)

Oligonucleotide Synthesis: A Practical Approach (Gait, M.J., Ed.) pp 35-81, IRL Press, Oxford). Site-directed mutagenesis was performed by using the method of Taylor, J.W. *et al.* (1985) Nucleic Acids Res. 13:8749-8764). Plasmid DNA was prepared from *E. coli* JM105 and purified by two successive centrifugation steps with cesium chloride density gradients.

BHK cells were grown in Dulbecco's modified essential medium (DMEM) with 10% fetal bovine serum to approximately 10^7 cells per 10-cm dish and were subsequently transfected with 10μg of plasmid by the calcium phosphate co-precipitation technique described by Searle, P.F. *et al.* (1985) Mol. Cell. Biol. 5:1480-1489). After 24 hours, the medium was changed to DMEM containing 100 μM methotrexate (MTX) and surviving cells were serially selected to 500 μM MTX. In some experiments, cells were selected immediately with 500 μM MTX. Large scale roller bottle cultures were initiated by seeding approximately 5 x 10^7 cells into each 850 cm^2 roller bottle containing 100 mL of DMEM-MTX. Cultures were induced at 80% confluency by the addition of $ZnSO_4$ to the medium to a final concentration of 0.08 mM. The medium was harvested 40 hours later.

Immune-precipitation and Western Blotting. Immune-precipitation of cell culture medium and cell lysates was performed by the method of Van Oost, B.A. *et al.* (1986) Biochem. Cell Biol. 64:699-705). Precipitates were analyzed by electrophoresis on 12% polyacrylamide gels in the presence of $NaDodSO_4$ (Laemmli, U.K. (1970) Nature (London) 227:680-685), followed by blotting onto a nitrocellulose membrane. The blot was incubated in PBS containing 0.1 mg/ml gelatin, then treated with goat anti-hTF antiserum (250-fold dilution in PBS), and finally developed with an alkaline phosphatase-conjugated, rabbit anti-goat IgG antibody according to the supplier's instructions.

Amino Acid Substitution. To incorporate 3-fluorotyrosine into the recombinant hTF/2N as a ^{19}F NMR probe, the culture medium was supplemented with D,L-m-fluorotyrosine (Sigma Chemical Company) at 16% of the concentration of L-tyrosine in the medium. The cells grew as well on this medium as on the medium lacking D,L-m-fluorotyrosine.

Isolation of Recombinant hTF/2N. Harvested culture medium was made 0.01% in phenylmethylsulfonyl fluoride to inhibit proteases and sufficient $Fe(III)(NTA)_2$ was added to saturate all transferrin in the medium. After stirring at room temperature, the solution was dialyzed for 24 hours versus cold running tap water, and then for a few hours versus Milli-Q purified water. Concentrated Tris-HCl buffer, pH 8.4 was added to a final concentration of 5 mM, the preparation was centrifuged to remove any debris, and was loaded onto a column (2.5 x 80 cm) of DEAE-Sephacel (Pharmacia) equilibrated with 10 mM Tris-HCl buffer, pH 8.4.

The column was then eluted with a linear gradient of NaCl (0 to 0.3 M) in the same buffer. Fractions showing a pink color were analyzed by $NaDodSO_4$-PAGE, and fractions containing the recombinant protein (Mr 37,000) were pooled. Such fractions also

contained bovine transferrin and albumin resulting from the fetal calf serum in the tissue culture medium. After concentration of the pooled fractions to 5 mL on an Amicon PM-10 membrane, the protein was chromatographed on a column (2.5 x 90 cm) of Sephadex G-75 Superfine (Pharmacia-PL Biochemicals) equilibrated with 100 mM ammonium bicarbonate.

Sometimes, a second chromatographic step through this column was necessary to resolve completely the hTF/2N from the bovine proteins. At this stage, the A_{465}/A_{410} was usually < 1.0, indicating the presence of a contaminating heme-protein (possibly hemopexin). The hTF/2N was finally purified to homogeneity by FPLC on a column (1 x 10 cm) of Polyanion SI (Pharmacia) using a linear gradient of NaCl (0 to 0.3 M) in 50 mM Tris-HCl, pH 8.0 over a period of an hour at a flow rate of lml/min. Fractions of l mL were collected. Two to four protein bands emerged from the column, depending on the iron-binding status of the protein.

$NaDodSO_4$-PAGE was performed with 5% to 12% gradient gels and urea-PAGE was performed according to a modification (Brown-Mason, A. and Woodworth, R.C. (1984) J. Biol. Chem. 259:1866-1873) of the Makey, D.G. and Seal, U.S. (1976) Biochim. Biophys. Acta 453:250-256 procedure. Electrofocusing was performed on a 0% to 50% sucrose gradient in a 110 mL glass column (LKB) with 0.8% Pharmalyte, pH 5 to 8 (Pharmacia). The column was prefocused overnight to a final current of 2 mA at 1000 V.

The protein sample in 0.2 mL was diluted with 5 mL of solution withdrawn from the middle of the gradient. The sample was then reinjected into the isodense region of the column and focusing was continued for 24 hours. The gradient was collected from the bottom of the column in 1.5 mL fractions. Individual fractions were analyzed for A_{280} and for pH. Fractions with maximum A_{280} were selected as representing the pIs of the apo- and iron-saturated proteins.

Iron was readily removed from the iron-protein by incubation in a buffer containing 1 mM NTA, 1 mM EDTA, 0.5 M sodium acetate, pH 4.9. The apo-protein was concentrated to a minimum volume on a Centricon 10 (Amicon), then diluted and reconcentrated twice with water and twice with 0.1 N KCl. The apo-protein had a tendency to precipitate in pure water, but redissolved readily in 0.1 M KCl. The apo-protein was made 10 mM in $NaHCO_3$ and titrated with a suitable concentration of $Fe(NTA)_2$ while monitoring the absorbance at 465 nm.

Quantitative Immunoassay of Recombinant hTF/2N. A competitive solid state immunoassay was used to assess the concentration of recombinant hTF/2N in the culture fluid and at various stages of the purification (Foster, W.B. *et al.* (1982) Thromb. Res. 28:649-661). Proteolytically-derived Fe-hTF/2N (Lineback-Zins, J. and Brew, K. (1980) J. Biol. Chem. 255:708-713) was radioiodinated (Fraker, P.J. and Speck, J.C., Jr. (1978) Biochem. Biophys. Res. Commun. 80:849-857) with Iodogen (Pierce Chemical Company) and used as the standard. The monoclonal anti-hTF antibody HTF-14 was used as the

probe (Bartek, J. *et al.* (1984) Folia Biol. (Prague) 30:137-140). This antibody recognizes only the amino-terminal lobe of hTF (Mason, A.B. *et al.* (1988) Br. J. Haematol. 68:392-393) and does not recognize bovine transferrin (Penhallow, R.C. *et al.* (1986) J. Cell. Physiol. 128:251-260).

Amino-terminal Sequence Analysis. The amino-terminal sequences of both the minor and major-forms of recombinant hTF/2N were determined on an Applied Biosystems 470A Protein Sequencer in the Given Analytical Facility at the University of Vermont.

Periodic Acid-Schiff Stain. The presence of oligosaccharides in the recombinant hTF/2N was determined by staining the protein with periodic acid-Schiff reagent (Fairbanks, G. *et al.* (1971) Biochemistry 10:2606-2617).

Nuclear Magnetic Resonance Spectroscopy. Proton and fluorine NMR spectra were obtained on the 5.872 Tesia Bruker WM NMR spectrometer in the Camille and Henry Dreyfus NMR Laboratory, Department of Chemistry, University of Vermont, operating in the Fourier transform mode with quadrature detection. An ^{19}F probe was provided by Dr. Christopher W. Allen of that department. For proton spectra, spectrometer settings were as described previously (Valcour, A.A. and Woodworth, R.C. (1987) Biochemistry 26:3120-3125). For ^{19}F spectra, the sweep width was 30,000 Hz, the acquisition time was 0.279 seconds, a receiver delay of 2.0 seconds intervened between acquisition and pulse of 15.0 μs (90°) and the sample was at 303°K. ^{19}F chemical shifts are relative to 0.1M trifluoroacetic acid in $^{2}H_2O$. Protein samples were 6 to 8 mg in 0.1 mL of 99.8 atom% $^{2}H_20$, and spectra were run on these samples in 0.1 mL capsules inserted into standard 5 mm NMR tubes containing $^{2}H_20$. Free induction decays of ^{19}F spectra were subjected to a line-broadening of 10 Hz prior to Fourier transformation.

RESULTS

Isolation of Human TF cDNA. Approximately 100,000 colonies of a human liver cDNA library (Prochownik, E.V. *et al.* (1983) J. Biol. Chem. 258:8389-8394) were screened by using a 24 base oligonucleotide to the 5' sequence of the human TF cDNA as a hybridization probe. A single positive colony was obtained. Extensive restriction enzyme mapping of the plasmid isolated from this clone agreed completely with the patterns predicted from the human TF cDNA isolated from the same library by Yang, F. *et al.* (1984) Proc. Natl. Acad. Sci. USA 81:2752-2756. DNA sequence analysis of the 5'- and 3'-termini of this clone confirmed that it was identical to the full-length clone isolated by Yang *et al.* All subsequent sequence analysis performed during the mutagenesis and subcloning of this cDNA conformed exactly to the sequence reported previously.

Vector Construction and Expression. Two translational stop codons and a unique *Hind*III recognition site were introduced into the linker region between the amino-

and carboxy-terminal domains of the hTF cDNA sequence by oligonucleotide-directed mutagenesis. The predicted translation sequence from this construct ends at Asp-337, according to the serum hTF numbering sequence (MacGillivray, R.T.A. *et al.* (1983) J. Biol. Chem. 258:3543-3553).

The expression vector pNUT (Palmiter, R.D. et al. (1987) Cell (Cambridge, MA) 50:435-443) contains a mouse metallothionein-1/human growth hormone gene fusion that has been shown to direct high levels of human growth hormone in transgenic mice (Palmiter, R.D. et al. (1983) Science (Washington, D.C.) 222:809-814). Important functional features of this vector include a mouse metallothionein-1 promoter to induce cDNA transcription in the presence of heavy metals, pUC18 sequences to allow replication and selection in *E. coli*, and a dihydrofolate reductase (DHFR) cDNA driven by the SV40 early promoter to allow selection in cell culture. The DHFR cDNA encodes a mutant form of the enzyme which has a 270-fold lower affinity for the competitive inhibitor methotrexate (MTX) (Simonsen, C.C. and Levinson, A.D. (1983) Proc. Natl. Acad. Sci. USA 80:2495-2499). This allows for the immediate selection of transfected cells in very high concentrations (0.5 mM) of MTX and abrogates the need for a recipient cell line that is deficient in DHFR.

To construct the expression vector pNUT-hTF/2N, the *Bam*HI-*Hind*III fragment from the bacterial expression vector was isolated (Figure 1). An *Hpa*II-*Bam*HI fragment from the original transferrin cDNA clone was also isolated (Figure 1). These two fragments were then ligated into M13mpl8 replicative form DNA that had been cut with *Acc*I and *Hind*III. Replicative form DNA from the resulting M13 phage was isolated, the insert released by cleavage with *Xba*I and *Hind*III, and the ends made blunt ended. These steps ensured that the fragment included the translational stop signals, retained the natural signal sequence for the protein, and was free of the dG/dC tail found in the original vector (Figure 1). This fragment was inserted into *Sma*I-cut pNUT, thus replacing the human growth hormone gene with a hTF/2N encoding cDNA, but leaving the transcriptional termination signal from the growth hormone gene intact. This plasmid was transfected into BHK cells and the resulting transformants were selected in the presence of MTX.

To analyze the mRNA transcripts produced by the transfected BHK cells, total RNA was electrophoresed on an agarose gel in the presence of formaldehyde (Maniatis, T. *et al.* (1982) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). After transfer to nitrocellulose, the blot was analyzed by using an oligonucleotide to the 3' untranslated region of the hGH gene as a hybridization probe. An inducible mRNA of approximately 1.4 kb was detected in the transfected cell line but not in mock-infected BHK cells (data not shown). This agreed with the predicted size of the hTF/2N mRNA, including the expected hGH 3' untranslated sequence and poly (A) tail.

To analyze the polypeptides produced by the transformed BHK cells, Western blot

analysis was performed both on cell lysates and the medium of various cell lines (Figure 2). Samples of BHK cells, BHK cells containing the hGH-pNUT plasmid, and BHK cells containing the hTF/2N-pNUT plasmid were grown in DMEM (BHK cells) or DMEM-MTX (BHK cells containing pNUT vectors). When the cells were reaching confluence, samples of medium were taken and cell lysates were prepared. These samples were incubated successively with goat anti-hTF antiserum and formalin-fixed S. aureus cells (Van Oost, B.A. *et al.* (1986) Biochem. Cell Biol. 64:699-705).

Bound proteins were eluted by incubation with $NaDodSO_4$, electrophoresed on a polyacrylamide gel, and transferred to a nitrocellulose membrane. The membrane was then incubated with goat anti-hTF antiserum and rabbit anti-goat immunoglobulin conjugated to alkaline phosphatase. When cell lysates or medium from BHK cells (Figure 2, lanes 1a and 1b) or BHK cells with hGH-pNUT plasmid (Figure 2, lanes 2a and 2b) were analyzed, only the expected goat immunoglobulin bands (Mr 25,000 and 50,000) from the original goat anti-hTF antibodies and a small amount of cross-reacting material were observed. However, an additional band of Mr 37,000 was observed in cell lysates (Figure 2, lane 3a) or medium (Figure 2, lane 3b) of the BHK cells containing the hTF/2N-pNUT plasmid. The molecular weight of this polypeptide chain is in excellent agreement with the molecular weight of the hTF/2N molecule (37,833) calculated from the amino acid sequence.

The homogeneity of the hTF/2N product indicates the successful removal of signal sequence as cell lysate and secreted samples comigrate on SDS-PAGE. The anti-serum appears to be highly specific for human TF species, since little bovine TF is apparent in the precipitates.

In large scale cultures of the hTF/2N cell line grown in roller-bottles, the concentration of hTF/2N in the medium was approximately 10-15 μg/ml as detected by radioimmunoassay.

Isolation and Characterization of Recombinant hTF/2N. Recombinant hTF/2N was purified by a three-step procedure that led routinely to an 80% yield of the major form of the protein, based on radioimmunoassay. The final purification on Polyanion SI led to quantitative resolution of the apo- and iron-saturated forms of both the minor (<5%) and major constituents of the protein (Figure 3, panel A), as corroborated by urea-PAGE (Figure 3, panel C). Note that on urea-PAGE the slowest moving bands are apo-hTF/2N and the faster moving bands are Fe-hTF/2N. SDS-PAGE gels (Figure 3, panel B) showed the major and minor forms of recombinant hTF/2N to be monodisperse, of equal molecular weight and the major component to be free of carbohydrate by PAS stain (data not shown).

In general these preparations appear to have better monodispersity than proteolytically derived hTF/2N (Lineback-Zins, J. and Brew, K. (1980) J. Biol. Chem. 255:708-713) (Figure 3). For example, the chromatographic peaks are more regular for the

former, and the number of bands on urea-PAGE is greater for the latter. Spectral ratios for the iron-saturated recombinant protein are typically A_{280}/A_{465} =21 and A_{465}/A_{410} = 1.38, which compare favorably with values for pure diferric transferrin isolated from human plasma. Titration of 3.68 A_{280} units of the apo-protein with $Fe(NTA)_2$ yields a slope corresponding to an E_{465}(mM) = 2.1 and gives for the apo-protein E_{280}(mM) = 38.8 (Figure 4), both reasonable values for a half-transferrin molecule (Lineback-Zins, J. and Brew, K. (1980) J. Biol. Chem. 255:708-713; Zak, O. *et al.* (1983) Biochim. Biophys. Acta 742:490-495). The pI's for the apo- and Fe-hTF/2N were 6.5 and 5.4, respectively.

Amino-terminal sequence analysis of both the minor and major forms of recombinant hTF/2N gave results identical to those found (MacGillivray, R.T.A. *et al.* (1983) J. Biol. Chem. 258:3543-3553) for holo-hTF from serum (Table 1).

The proton NMR spectrum of the recombinant protein (Figure 5) is very similar to that for the proteolytically-derived hTF/2N (Valcour, A.A. and Woodworth, R.C. (1987) Biochemistry 26:3120-3125), but the resonance lines are sharper for the recombinant protein. The ^{19}F NMR spectrum of the protein derived from a cell culture grown on medium supplemented with **m**-F-tyrosine (Figure 6) shows four well-resolved resonances, two possibly having an unresolved shoulder.

Table 1

Amino-Terminal Sequence of Human Transferrin and of the Recombinant Human Transferrin Amino-Terminal Half-Molecule[a]

Protein	Amino Acid Sequence	Reference
human serum transferrin	V-P-D-K-T-V-R-W-C-A-V-S-	MacGillirvray *et al.* (1983) (SEQ ID NO:5)
recombinant hTF/2N (major)[b, c]	V-P-D-K-T-V-R-W-X-A-V-S-	this report (SEQ ID NO:6)
recombinant hTF/2N (minor)[d]	V-P-D-K-T-V-	this report (SEQ ID NO:7)

[a]The recombinant hTF/2N sequences were determined on an Applied Biosystems 470A protein sequencer. Approximately 200 pmol of each sample was analyzed. [b]Twelve sequencer cycles were analyzed. [c]No residue was identified at cycle 9; however, cysteine residues were not modified prior to the analysis. [d]Six sequencer cycles were analyzed.

By using recombinant DNA technology, a hTF/2N molecule is produced that functions identically with the proteolytically derived species as judged by several independent criteria. This represents the first reported expression in a stable cell culture system of a functionally active form of this important iron transport protein.

The pNUT based hTF/2N construction described here produces high levels of recombinant protein without the need for a DHFR-deficient cell line or tedious resistance amplification procedures. BHK cells are well-suited for economical, large scale growth and we are currently examining their growth characteristics on micro-carrier supports in bioreactor vessels. By using either roller bottles or a fermentor with a capacity of several liters, we can easily produce sufficient recombinant protein even for techniques such as NMR that traditionally have required a high concentration of protein.

The minor form of recombinant hTF/2N isolated on Polyanion SI migrates more slowly than the major form on urea-PAGE (Figure 3, panel C), but at the same rate on SDS-PAGE (Figure 3, panel B). Thus, the apparent molecular weights are the same but the relative degrees of unfolding in 6 M urea differ. Note that the proteolytically-derived apo-hTF/2N shows even faster migrating species in 6 M urea (Figure 3, panel C, fractions g and h).

Contamination of apo-hTF/2N with Fe-hTF/2N and vice versa on these gels arises from the method of pooling FPLC fractions, from some loss of bound iron on the urea gel and from binding of contaminating iron during workup of the FPLC samples. Identical N-terminal sequences (Table 1) show that the signal peptide has been removed from both minor and major forms of the recombinant protein. As in hTF/2N from human serum (Lineback-Zins, J. and Brew, K. (1980) J. Biol. Chem. 255:708-713), the recombinant hTF/2N is non-glycosylated. The cause of the difference between major and minor forms of hTF/2N is unknown at present. The minor form has never represented more than 5% of the total recombinant protein and is usually less than 1%. Thus, the goal of isolating a monodisperse recombinant hTF/2N (the major form) has been achieved.

The iron binding behavior, pIs, migration on NaDodSO$_4$-PAGE and urea-PAGE and proton NMR spectra of the recombinant hTF/2N match reasonably well those of the hTF/2N derived from amino terminal monoferric hTF by proteolysis with thermolysin (Lineback-Zins, J. and Brew, K. (1980) J. Biol. Chem. 255:708-713; Valcour, A.A. and Woodworth, R.C. (1987) Biochemistry 26:3120-3125), except as noted above. The major form of the recombinant protein shows a higher degree of monodispersity (Figure 3) and its proton NMR spectrum shows sharper resonance lines than does the proteolytically derived hTF/2N. There has been insufficient minor form for analysis by NMR.

Previous studies of the incorporation of m-fluorotyrosine into alkaline phosphatase from *E. coli* have established the efficacy of ^{19}F NMR for specifically probing the tyrosyl residues in a protein (Sykes, B.D. *et al.* (1974) Proc. Natl. Acad. Sci. USA 71:469-473; Hull, W.E. and Sykes, B.D. (1974) Biochemistry 13:3431-3437). Incorporation of m-F-

tyrosine into the recombinant hTF/2N proves that selective amino acid substitution is possible in this cell culture system and gives access to a specific NMR probe of tyrosyl side chains. This preparation behaves in all respects like the non-modified protein as described above for the non-substituted recombinant. When the cell culture conditions have been optimized to achieve higher levels of incorporation, changes in the ^{19}F NMR spectrum on addition of paramagnetic and diamagnetic metals and on changes in pH can be useful in studying the tyrosyl residues specifically involved in metal binding. Incorporation of selectively deuterated aromatic amino acids can allow dissection of the aromatic region of the proton NMR spectrum of the protein in similar fashion to the studies on lysozyme from Japanese quail (Brown-Mason, A. *et al.* (1981) J. Biol. Chem. 256:1506-1509).

II. Production of Recombinant Transferrin Half- Molecule Comprising Carboxy Terminal Lobe.

An *Eco*RI restriction fragment including the coding sequence for the carboxy lobe of hTF was isolated from the full length hTF cDNA and then used as a template for PCR-directed mutagenesis (Figure 2). Two oligonucleotides were synthesized to be used as PCR primers. Oligo 1 encodes a *Sma*I recognition site, followed by sequence encoding the natural signal sequence of hTF, followed by sequence matching the coding sequence for amino acids 334-341. The second oligonucleotide matches the complement of the 3' nontranslated region of the hTF cDNA and introduces a *Sma*I recognition sequence 3' to the normal translation termination site (nucleotides 2125-2127 using the numbering system of Yang, F. *et al.* (1984) Proc. Natl. Acad. Sci. USA 81:2752-2756). Twenty-five rounds of PCR amplification using Taq polymerase (Perkin Elmer) resulted in the desired DNA fragment which splices the natural signal sequence of hTF to the C lobe coding sequence. This fragment was then digested with *Sma*I and ligated with the large *Sma*I fragment of pNUT as for the hTF/2N expression studies.

III. Production of Recombinant Full Length Transferrin.

The coding sequence for human serum transferrin was assembled from restriction enzyme digestion fragments derived from the full-length cDNA clone isolated from a human liver library described above. Since the parental plasmid (pKT-218) of the original clone had a limited number of unique restriction enzyme recognition sites, a series of cloning steps was required to introduce the coding sequence into a convenient vector. This process was initiated by cloning a HpaII/BamHI fragment from the 5' end of the cDNA into the vector pUC 18 (Messing, J. (1983) Meth. Enzymol. 101:20-28). The resulting plasmid was digested with BamHI and HindIII and a BamHI/HindIII fragment

from the human transferrin cDNA was cloned adjacent to the initial fragment. The resulting plasmid was then digested with HindIII and PstI and a final HindIII/PstI fragment from the 3' end of the transferrin cDNA was cloned to complete the assembly of the full-length coding sequence. Digestion of the resulting plasmid with SacI and SphI released the full-length coding sequence as a single restriction fragment which was subsequently made blunt using T4 DNA polymerase and dNTPs and then cloned into the large SmaI fragment of pNUT (Palmiter *et al.* (1987) Cell 50:435-443) as described for the N-and C-terminal transferrin half-molecule coding sequences.

Plasmid DNA was prepared from *E. coli* JM105 and purified by two successive centrifugation steps with cesium chloride gradients. Baby hamster kidney (BHK) cells were grown in Dulbecco's Modified Eagles' medium-Ham's F-12 nutrient mixture (DMEM-F-12) (Gibco; Sigma) with 10% fetal bovine serum to approximately 10^7 cells per 100 mm dish and were subsequently transfected with 10 μg of plasmid by the calcium phosphate coprecipitation technique described by Searle *et al.* (1985) Mol. Cell Biol. 5:1480-1489. After 24 hours, the medium was changed to DMEM-F-12 containing 500 μ M methotrexate to select the plasmid containing cells. Once selected, the cells were serially passaged at approximately 80% confluency with phosphate buffered saline containing EDTA (0.2 gm/l) to five 100-mm dishes, then to five T-175 flasks and finally to five expanded surface roller bottles (200 ml each). At the T-175 passage, a serum substitute, Ultroser G (Gibco), at a level of 1% was used in place of fetal calf serum in DMEM-F-12 lacking phenol red.

It was found that once production levels were high (approximately 100 μg/ml of medium), medium without Ultroser G™ could sustain production of recombinant protein for at least two passages. This greatly simplified the isolation of the expressed full-length recombinant human serum transferrin. To isolate the recombinant protein, harvested culture medium is made 0.01% with respect to phenylmethanesulfonyl fluoride and sodium azide to inhibit proteases and bacterial growth respectively. Sufficient Fe^{3+} (nitrilotriacetic acid)$_2$ is added to saturate the transferrin present. The medium is reduced in volume to <10 ml and the transferrin is purified by passage over an anion exchange column (Polyanion SI, 1 x 10 cm) as described for the recombinant amino terminal human transferrin half-molecule above.

The isolated recombinant full-length human serum transferrin displays some heterogeneity on this column attributed to variation in the glycosylation pattern. The protein is monodisperse on NaDod SO_4-polyacrylamide gel electrophoresis and has a spectrum and spectral ratios which are comparable to purified human serum transferrin.

IV. Production of Mutant Transferrins.

Substitution mutants are designated using the conventional single letter amino acid symbol of the wild type (native) residue, followed by the positional number of the replacement in the primary sequence, (where valine of the mature protein is designated position 1) followed by the symbol for the replacement residue. For example, a mutant in which aspartic acid residue at position 63 is replaced by a serine residue would be designated D63S.

The production of hTF/2N mutants was accomplished by two techniques. A D63S substitution was prepared using the method of Nelson, R.M. and Long, G.L. (1989) Analyt. Biochem. 180:147-151. Briefly, a HpaII/BamHI fragment from the 5' end of the hTF/2N coding sequence was subcloned into pUC18 and then used as a template for a two step PCR-based mutagenesis procedure. The resulting DNA fragment was then recloned into M13mp18 and the sequence of the mutant construction was confirmed by dideoxy sequence analysis. The fragment was then released from the double stranded form of the sequencing vector by digestion with XbaI and BamHI and then ligated to a BamHI/HindIII fragment from the original hTF/2N construction to produce a full length D63S-hTF/2N coding sequence, the fidelity of this splicing was confirmed by restriction digestion analysis and was subsequently cloned into pNUT as before.

The substitution mutants G65R, D63C, K206Q and H207E were produced by subcloning the entire hTF/2N coding sequence into M13mp18, which was then used as a template for oligonucleotide-directed mutagenesis (Zoller, M.J. and Smith, M. (1983) Meth. Enzymol. 100:458-500) using the dut$^-$, ung$^-$ selection procedure (Kunkel, T.A. (1985) Proc. Natl. Acad. Sci. USA 82:488-492). Following mutagenesis, the entire coding sequence for the mutant sequences was confirmed by dideoxy sequence analysis using sequencing primers targeted along the length of the coding sequence at 250 bp intervals. The desired coding sequences were then released by restriction digestion, made blunt and inserted into pNUT as before.

pNUT plasmids have been constructed containing the cDNA a) for full-length human serum transferrin (hTF) and b) for various site-directed mutants of the amino-terminal half-molecule (hTF/2N). These mutants include 1) D63S patterned on the naturally occurring mutation found in the C-terminal half of human melanoferrin, b) G65R patterned on the naturally occurring mutant found in the C-terminal half of hTF from a patient in England, c) K206Q based on the wild type mutation in the C-terminal half of ovotransferrin (oTF) from hen's egg white, d) H207E based on the wild type mutation in human lactoferrin (hLTF) and e) D63C as an attempt to change the metal selectivity of the iron binding site. All of these constructions have been expressed in stable transformants of baby hamster kidney cells in 10 to 100 mg amounts of recombinant protein. In addition pNUT plasmids have been constructed containing the full length cDNA for oTF and

chimeric cDNAs for hTF/2N-oTF/2C and oTF/2N-hTF/2C.

Characteristics of the site-directed mutants include: the D63S mutant does bind iron (contrary to speculations in the literature) but much less avidly than the wild type protein. For instance, this mutant loses its bound iron on electrophoresis in PAGE gels containing 8 M urea, whereas the wild type retains its bound iron. The maximum in the visible spectrum lies at 422 nm in contrast to that or the wild type at 470 nm. The G65R mutant binds iron less tightly than does the wild type and has a visible maximum at 470 nm. The K206Q mutant binds iron much more avidly than does the wild type, as does its model, oTF/2C. Whereas the red color of the wild type iron protein disappears very rapidly in 0.5 M acetate buffer at pH 4.9, containing 1 mM each of EDTA and NTA, the mutant loses no color at all and requires pH 4 and 1 mM deferoxamine to release its bound iron. The apo-mutant appears to rebind iron more slowly than the wild type protein. The visible maximum lies at 460 nm for this mutant. The H207E mutant also binds iron more avidly than does the wild type.

The full length recombinant hTF runs at the same rate as the serum-derived protein on SDS-PAGE.

V. Removal of Glycosylation Sites from hTf.

Human serum transferrin contains two N-linked oligosaccharides, at Asn-413 and Asn-611 (MacGillivray *et al.* (1982) PNAS USA 79:2504-2508), corresponding to AAT and AAC codons in the cDNA sequence, respectively (Yang *et al.* (1984) Proc. Natl. Acad. Sci. USA 81:2752-2756). These codons were converted to GAT and GAC by oligonucleotide-directed mutagenesis using the dut$^-$ and ung$^-$ method (Kunkel, T.A. (1985) Proc. Natl. Acad. Sci. USA 82:488-492). The mutagenic oligonucleotides:

5'-GCAGAAAACTACGATAAGAGCGATAAT-3' (SEQ ID NO:3)

5'-CTATTTGGAAGCGACGTAACTGACTGC-3' (SEQ ID NO:4)

(the mutated codons are underlined) were synthesized on an Applied Biosystems 391 DNA sunthesizer, and were purified by reverse-phase chromatography using a SEP-PAK (Waters) column (Atkinson, T. and Smith, M. (1984) Oligonucleotide Synthesis: A Practical Approach (Gait, M.J., Ed.) pp 35-81, IRL Press, Oxford).

The template for the mutagenesis was a plasmid containing the DNA coding sequence for the C-lobe of transferrin cloned into pUC named pUC2-3; as shown in Figure 8, this plasmid contains a *Not*I site in the interlobe bridge coding region and a *Sma*I site in both the 5' and 3' untranslated regions. Each of the two mutagenic oligonucleotides was used separately to introduce the desired mutations into pUC2-3; the resulting plasmids

were Tf-N413D and Tf-N611D (see Figure 8). The presence of the mutated codons was confirmed by DNA sequence analysis (Sanger, F. *et al.* (1977) Proc. Natl. Acad. Sci. USA 74:5463-5467). Each plasmid was cut with *Acc*I and *Stu*I, the DNA fragments were separated by agarose gel electrophoresis, and the fragments containing the mutated residues were recovered from gel slices using GENECLEAN (Bio101, La Jolla, CA). The fragments were then ligated back into the *Acc*I site of the full-length transferrin cDNA clone in pUC19. The structure of the final construction, hTf(N/D), was confirmed by restriction mapping and DNA sequence analysis. The transferrin cDNA was then released with *Sac*I and *Sph*I; the ends were made blunt by treatment with the Klenow fragment of DNA polymerase I in the presence of dNTPS and ligated directly into pNUT restricted with S*ma*I (Palmiter, R.D. *et al.* (1987) Cell 50:435-443) as described for the N- and C-terminal transferring half-molecule coding sequences. The correct orientation of the pNUT-hTf(N/D) clone was confirmed by restriction-endonuclease mapping.

The pNUT-hTf (N/D) clone was then treated in the same manner as described for the full recombinant length transferrin. The resulting transformations were selected using 500μM MTX.

The isolated hTf N413D/N611D mutant protein was monodisperse on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and had a spectrum and spectral ratios similar to that of serum-derived hTf. However, the hTf N413D/N611D mutant migrates slightly faster than serum-derived hTf.

VI. Cell-Binding Experiments.

HeLa S_3 cells were the generous gift of Dr. Joan Moehring (Department of Microbiology, University of Vermont College of Medicine). Cells were routinely grown in DMEM-F-12 containing 10% newborn calf serum. Prior to beginning a binding experiment, the cells were harvested with Versene, and taken up in Joklik's minimum essential medium-20 mM Hepes-2% BSA (JMEM-BSA). Endogenous bovine transferrin was removed from the HeLa cells by incubation for 10 min at 37°C at a 5-fold dilution with JMEM-BSA. After centrifugation of the cells and removal of the supernatant, this procedure was repeated twice. The cells were then incubated for an additional 10 min in the presence of 10 mM NH_4Cl to inhibit the removal of iron from transferrin (Morgan (1981) Biochim. Biophys. Acta 642:119-134; Harding & Stahl (1983) Biochem. Biophys. Res. Comm. 113:650-658; Rao *et al.* (1983) FEBS Lett. 160:213-216; Klausner, *et al.* (1983) J. Biol. Chem. 2578:4715-4724; Mason *et al.* (1987) Biochem J. 245:103-109). Removal of the endogenous transferrin is somewhat superfluous since bovine transferrin has a very low affinity for human receptors and would not effectively compete with human transferrin in the binding studies (Penhallow, R.C. *et al.* (1986) J. Cell. Physiol. 128:251-260). For each diferric hTf sample to be tested, cell suspensions (300 μL

containing ~2.2 x 10^6 cells) were added to eight different Omnivials containing between 3 and 80 pmol of radiolabeled diferric transferrin. An identical set of vials was set up containing a 100-fold excess of unlabeled Boehringer Mannheim diferric transferrin to determine the amount of nonspecific binding. After 30 min of incubation at 37°C with gentle shaking, portions of the cell suspension (3 x 100 µL) were pipetted into microfuge tubes containing 0.9 mL of ice-cold JMEM-BSA over 300 µL of dibutyl phthalate and centrifuged for 2 min in a Beckman microfuge. The aqueous and organic phases were aspirated to just above the cell pellet. The bottom of the tube containing the cell pellet was released by a hot wire into a plastic tube (12 x 75 mm) and assayed for radioactivity. A second approach involved competing six different amounts (4-120 pmol) of each of the different hTf samples against a constant amount (6.4 pmol) of radioiodinated Boehringer Mannheim hTf. The program LIGAND was used to analyze the data from both types of experiment assuming a single class of binding sites in each case (Munson & Rodbard, (1980) Anal. Biochem. 107:220-239).

In order to test the functional integrity of the five different hTf samples, equilibrium binding studies were undertaken using two different approaches as discussed above. First, each hTf sample was radioiodinated, and direct binding to HeLa S_3 cells was measured in the presence and absence of a 100-fold excess of unlabeled Boehringer Mannheim Fe_2hTf. In all instances, the amount of nonspecific binding was very low, less than 5% of the specific binding. The data from the equlibrium binding experiment were analyzed by the nonlinear curve-fitting program of Munson and Rodbard to determine the affinity and binding site number for each TF (Munson & Rodbard, (1980) Anal. Biochem. 107:220-239). A typical data set is presented in Table 2. The results show that all of the transferrins bound with approximately the same affinity and to the same extent.

The second approach involved competing different amounts of each of the transferrins (unlabeled) against a constant amount of radioiodinated Boehringer Mannheim diferric hTf. The results of a typical data set from this approach are presented in Table IIIB. The two experiments shown were done on different days which probably accounts for the difference in the number of binding sites per cell observed. In many experiments over a number of years (Penhallow, R.C. *et al.* (1986) J. Cell. Physiol. 128:251-260), between 0.8 and 2 x 10^6 sites/cell have been found, which probably reflects the metabolic state of the cells. Binding of the recombinant Tf samples is indistinguishable from binding of the commercially available Tf samples.

Table 2[a]

transferrin	Kd'[b](nM)	TF/cell[c] x 10^{-6}	N[d]
(A) Results of Equilibrium Binding Experiments in which Binding of Radioiodinated Diferric Transferrin to HeLa S_3 Cells Was Directly Measured			
recombinant glycosylated	31.3 ± 3.6	2.09 ± 0.14	0.004 ± 0.003
recombinant nonglycosylated	23.4 ± 2.5	1.96 ± 0.13	0.013 ± 0.003
Boehringer Mannheim	17.8 ± 2.3	1.31 ± 0.08	0.019 ± 0.003
Sigma	19.9 ± 1.5	1.76 ± 0.14	0.009 ± 0.004
Scipac	22.5 ± 2.9	1.76 ± 0.09	0.008 ± 0.002
(B) Results of Equilibrium Binding Experiments in which Six Different Amounts of Unlabeled Transferrin Were Competed against a Constant Amount of Boehringer Mannheim Radioiodinated Transferrin			
recombinant glycosylated	22.6 ± 2.4	0.99 ± 0.03	0
recombinant nonglycosylated	19.8 ± 7.4	0.91 ± 0.07	0
Boehringer Mannheim	29.7 ± 1.6	1.00 ± 0.10	0.015 ± 0.006
Sigma	19.6 ± 9.7	0.79 ± 0.07	0.018 ± 0.005
Scipac	30.0 ± 1.8	1.04 ± 0.10	0.013 ± 0.006

[a]In (A), 7482 cpm bound (3.28×10^5 TF/cell) in the absence of competitor. A total of 44 cpm bound in the presence of 100-fold excess of unlabeled competitor. [b]In both experiments, K_d' denotes the apparent equilibrium binding constant. [c]TF/cell denotes the number of TF molecules bound per cell. [d]N denotes the ratio of nonspecifically bound to free transferrin.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.